Gene 639 (2018) 77–84

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

LncRNA-LINC00460 facilitates nasopharyngeal carcinoma tumorigenesis through sponging miR-149-5p to up-regulate IL6

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ARTICLE INFO

Keywords:
LINC00460
miR-149-5p
IL6
Nasopharyngeal carcinoma (NPC)
Tumorigenesis

ABSTRACT

Long non-coding RNAs (lncRNAs) have played crucial roles in various cancers, including nasopharyngeal carcinoma (NPC). In our study, we focused on the biological function and clinical significance of lncRNA LINC00460 in NPC. It was indicated that LINC00460 was markedly increased in NPC tissues and cells compared to their corresponding controls. Silencing LINC00460 was able to suppress NPC cell growth in vitro while overexpressing LINC00460 reversed this process. Moreover, in vivo tumor xenografts were established using CNE-1/SUNE-1 cells to detect the function of LINC00460 in NSCLC tumorigenesis. Rescue assay was performed to further confirm that LINC00460 contributed to the progression of NPC through regulating miR-149-5p/IL6 signal pathway. In conclusion, we have uncovered that LINC00460 could be regarded as a novel prognostic biomarker and therapeutic target in NPC diagnosis and treatment.

1. Introduction

Nasopharyngeal carcinoma (NPC), occurred in the epithelial lining of the nasopharynx and mainly prevalent in Southeast Asia and Southern China, is one of the most common types of head and neck tumors (Hu et al., 2012; Song and Yin, 2016). Currently, radiochemotherapy and radiotherapy are the primary methods for treatment of NPC, but a number of patients invariably grow to neck and/or distant metastasis, and the prognosis still remains poor due to its high metastasis (Jiang et al., 2016; Song and Yin, 2016). Hence, further investigations for the molecular mechanisms of NPC tumorigenesis and more valid therapeutic strategies are authoritatively required.

Long non-coding RNAs (lncRNAs) are non-protein-coding transcripts whose length are above 200 nucleotides (nt). Increasing studies have reported that lncRNAs involved in hands of physiological and pathological processes through regulating gene expression at the transcriptional, post-transcriptional, and epigenetic levels. Up to date, several cancer-related lncRNAs are found to involve in tumor growth, migration, invasion and metastasis, and have been revealed as underlying alternative therapeutic targets and biomarkers for human cancers (Fang et al., 2016; Sun et al., 2016a, 2016b, 2016c, 2016d, 2016e; Sun et al., 2016f). Recently, several lncRNAs, including EWSAT1 (Song and Yin, 2016), LOC100129148 (Sun et al., 2017), PCAT7 (D. Liu et al., 2017; Y. Liu et al., 2017), and CCAT1 (Wang et al., 2017) are related to NPC tumorigenesis. Nevertheless, the detail molecular mechanism during NPC tumorigenesis is still sustain unclear.

In the current study, we characterized a new reported lncRNA, LINC00460, it is located at chromosome 13q33.2 and is transcribed into a 935 nt in length transcript. LINC00460 was significantly upregulated in NPC tissues in comparison to their corresponding non-tumor tissues. We also found that overexpression of LINC00460 was closed associated with poor prognosis in NPC patients. Silence of LINC00460 was found to repress NPC cells proliferation both in vitro and in vivo. Additionally, our data revealed that LINC00460 could function as an oncogene in part through up-regulating the miR-149-5p targeted gene IL6 by functioning as a competitive endogenous RNA (ceRNA) for miR-149-5p in NPC. Our results make clear that LINC00460 might represent a new pointer for poor prognosis and is a feasible biomarker for diagnosis and treatment of NPC patients.

Abbreviations: lncRNAs, long noncoding RNAs; NPC, nasopharyngeal carcinoma; EMT, epithelial-to-mesenchymal transition; ceRNA, competing endogenous RNAs; miR-149-5p, hsa-miRNA-149-5p; ANOVA, one-way analysis of variance; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IHC, immunohistochemistry; GBC, gallbladder cancer; RCC, renal cell carcinoma

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http://dx.doi.org/10.1016/j.gene.2017.10.006
Received 28 June 2017; Received in revised form 30 September 2017; Accepted 4 October 2017
Available online 05 October 2017
0378-1119/

2. Materials and methods

2.1. Ethical statement and tissue collection

50 patients diagnosed as NPC were involved in this study. All histologic diagnoses were conducted by the pathology department at the Renmin Hospital of Wuhan University. Normal nasopharynx epithelial tissues were obtained from non-tumor adjacent tissues. Informed consent was received from all subjects. All experimental protocols were permitted by the Ethics Committee at the Renmin Hospital of Wuhan University.

2.2. Cell lines and plasmids

Six human NPC cell lines (SUNE-1, CNE-1, HNE-1, CNE-2, C666-1 and HONE-1) were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) (Kibbutz BeitHaemek, Israel). The human immortalized nasopharyngeal epithelial cell line NP69 was cultured in keratinocyte/serum-free medium (Invitrogen) supplemented with bovine pituitary extract. All cells were cultured at 37 °C in a 5% CO_2 atmosphere and maintained in 10% FBS (Kibbutz BeitHaemek, Israel). Plasmid pcDNA3.1-IL6 and pcDNA3.1-LINC00460 were prepared by ourselves. RNAi sequence: **LINC00460**: sh-1 target CGTGGGAAAGAAGACGCATTCTGAA; sh-2 target CCTGAGCCATCCACTTCAAAGTATT; sh-3 target CACGCCTCTGAAATGGTGACAATAA.

2.3. Western blot analysis

Western blot was conducted by the protocol described previously (Sun et al., 2015a, 2015b; Sun et al., 2016a, 2016b, 2016c, 2016d, 2016e). Briefly, cells were harvested, rinsed with PBS and lysed in lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM MgCl2, 0.5% NP-40, 1 mM Na3VO4, 1 mM NaF, protease inhibitors cocktail). Cell lysates were separated on SDS–polyacrylamide gel, transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories) and immunoblotted using the following primary antibodies. Primary antibody of IL6, GAPDH (Santa Cruz, CA) were used for WB analysis.

2.4. qRT-PCR

Total RNA was extracted from cultured cells or frozen tissues by the TRIzol reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. The isolated RNA was reverse transcribed to cDNA using a PrimeScript RT reagent Kit (Takara, Dalian China). Real-time PCR analysis were conducted using SYBR Green (Takara, Dalian China). Results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers used were as follows: LINC00460 forward, 5′-ACAGCATGAGCCAGGACATC-3′, and reverse, 5′-GAAAGCTGCAACATGCTCCC-3′; GAPDH forward, 5′-AGCCACATCGCTCAGACAC-3′, and reverse, 5′-GCCCAATACGACCAAATCC-3′. qRT-PCR were performed on an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). $2^{-\Delta\Delta Ct}$ method was used to quantify LINC00460.

2.5. Cell viability analysis and colony formation assay

Viability of cells transfected with indicated shRNA or plasmid constructs was determined by trypan blue dye exclusion and CCK-8 assay (Sun et al., 2016a, 2016b, 2016c, 2016d, 2016e; Xi et al., 2016). Colony formation assay was performed with CNE-1 and SUNE-1 cells transfected with sh-NC and sh-LINC00460 for 14 days followed by crystal violet staining.

2.6. Luciferase reporter assays

Luciferase reporter assays was conducted as described previously (Stiuso et al., 2015; Catapano et al., 2016; F. Li et al., 2016; J. Li et al., 2016; Miao et al., 2016; Sun et al., 2016a, 2016b, 2016c, 2016d, 2016e; Wang et al., 2016; Xi et al., 2016; Sun et al., 2016f).

2.7. Biotinylated RNA pull-down assay

The biotinylated RNA pull-down assay was conducted as described previously (Tsai et al., 2010; Hung et al., 2014). Biotin-labelled RNAs was in vitro transcribed with AmpliScribe T7-Flash Biotin-RNA Transcription Kit (Epicentre), treated with RNase-free DNase I and purified with an RNeasy Mini Kit (Qiagen). The lambda transcript was generated with the control plasmid provided by the Transcription Kit. To form the proper secondary structure, biotinylated RNA supplied with RNA structure buffer (10 mM Tris pH 7, 0.1 M KCl and 10 mM MgCl2) was heated to 90 °C for 2 min, incubated on ice for 2 min and then shifted to room temperature (RT) for 20 min. The RNA was then mixed with cell nuclear extract or purified proteins and incubated at RT for 1 h, followed by incubating with Streptavidin Mag Sepharose (GE Healthcare) at RT for 1 h. After subsequent washes, the pull-down complexes were analyzed by standard western blot technique.

2.8. Tumor formation in nude mice

5 weeks old male BALB/c nude mice were maintained under specific pathogen-free conditions and were manipulated according to protocols approved by the Wuhan Medical Experimental Animal Care Commission. CNE-1/SUNE-1 cells were stably transfected with sh-LINC00460 (namely, sh-460) or empty vector using Lipofectamine 2000 (Invitrogen). After 48 h, the cells were collected and harvested at a concentration of 2×10^7 cells/mL. 100 μL of the cell suspension was subcutaneously injected into a single side of the posterior flank of each mouse. Tumor volumes and weights were tested every 4 days in mice from the sh-NC (six mice) or sh-460 (six mice) groups. The tumor volumes were measured (length × width^2 × 0.5). 36 days after injection, the tumors were isolated from all of the animals and used for further analysis.

2.9. Statistical analysis

The results were analyzed using GraphPad Prism (GraphPad Software Inc., La Jolla, CA) and the SPSS 23.0 software (SPSS, Chicago, IL, USA). The significance of differences was assessed using Student's *t*-test or a one-way ANOVA. All data are expressed as the means ± SEM. $P < 0.05$ indicates a significant difference.

3. Results

3.1. Up-regulated LINC00460 is found in human NPC tissues and cells, and correlates with poor prognosis

Using the bioinformatics tool lncRNAtor (http://lncrnator.ewha.ac.kr/index.htm), we analyzed RNA-Seq data (from TCGA: The Cancer Genome Atlas) for lncRNAs from Head and Neck squamous cell carcinoma tissues (n = 341). Results showed that LINC00460 was up-regulated approximately 5.5-fold in NPC tissues (Fig. 1A). Next, we analyzed LINC00460 expression in 300 NPC tissue samples and 21 normal tissue samples from TCGA. Results indicated LINC00460 were remarkably up-regulated in NPC tissues ($P < 0.05$) (Fig. 1B). Then, we assessed LINC00460 expression in 50 pairs of NPC tissues and adjacent normal tissues. Results showed that LINC00460 levels in tumors were markedly higher in 90% (45/50) of the samples in comparison to those of in corresponding normal counterparts, with a 3.78-fold up-regulation ($P < 0.05$) (Fig. 1C). LINC00460 was also found to be up-regulated in